Competition mechanisms of lactic acid bacteria and bifidobacteria: Fermentative metabolism and colonization.

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Abstract

Enzyme activities (α- and β-glucosidases, α- and β-galactosidases and β-fructofuranosidase) and organic acid production of four strains of lactic acid bacteria (LAB; *Streptococcus thermophilus* STY-31, *Lactobacillus delbrueckii* subsp. *bulgaricus* LBY-27, *Lactobacillus casei* LC-01 and *Lactobacillus acidophilus* LA-5) and *Bifidobacterium lactis* BB-12 were tested on milk and MRS fermentation broth with glucose, lactose or fructooligosaccharides (FOS) as carbon source. The highest β-galactosidase activity was found in *L. acidophilus* growing on milk. As compared to milk, α-glucosidase activity was increased with FOS by *B. lactis*, *L. acidophilus* and *L. casei*. The analysis of organic acids and short chain fatty acids in the medium growth showed that lactate and acetate were the major fermentation metabolites produced by LAB and bifidobacteria, respectively. However, a metabolic shift toward more acetate and formate production, at the expense of lactate production, was observed during growth of *L. casei* on FOS. When grown on FOS as sole carbon source, *L. acidophilus* showed the highest production of lactate among the species tested. In addition, *L. acidophilus* demonstrated resistance to colonization against the intestinal pathogens *Escherichia coli* and *Salmonella enterica* in competition assays.

Keywords: Lactic acid bacteria; Bifidobacteria; Fructooligosaccharides; Galactosidases; Glucosidases

1. Introduction

Fermented milk products are the most popular means of delivering probiotic bacteria in food. Among them, strains of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium lactis* predominate in commercial probiotic products (Gueimonde, Delgado, Mayo, Ruas-Madiedo, Margolles, & De los Reyes-Gavilán, 2004; Masco, Huys, De Brandt, Temmerman, & Swings, 2005; Tabasco, Paarup, Janer, Peláez, & Requena, 2007). Prebiotics are closely associated with probiotics given that they are defined as fermentable substrates that selectively stimulate the growth and/or activity of the intestinal bacteria that contribute to health and well-being (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). Three types of carbohydrates are usually classified as prebiotics, namely inulin-type fructans, trans-galacto-oligosaccharides, and lactulose (Gibson et al., 2004). Fructooligosaccharides (FOS) are oligosaccharides of D-fructose units linked by a β-(2, 1) glycosidic linkage, with a terminal glucose or fructose unit. Not all probiotic bacteria commonly used as food ingredients are able to metabolize FOS (Shene, Mardones, Zamora, & Bravo, 2005). A variety of bifidobacteria and lactobacilli have been found to ferment inulin-type fructans (Barrangou, Azcarate-Peril, Duong, Conners, Kelly, & Klaenhammer, 2006; Goh, Zhang, Benson, Schlegel, Jong-Hwa, & Hutkins, 2006; Van der Meulen, Makras, Verbrugghe, Adriany, & De Vuyst, 2006). *B. lactis* BB12 has been found to first utilize the shorter oligosaccharides in a FOS-mixture making use of a cytoplasmic β-fructofuranosidase (Janer, Arigoni, Lee, Peláez, & Requena, 2005). Similarly, *L. acidophilus* hydrolyses FOS via intracellular hydrolysis (Barrangou, Altermann, Hutkins, Cano, & Klaenhammer, 2003). On the other hand, the β-fructosidase of *L. casei* is cell-wall associated (Goh, Lee, & Hutkins, 2007). Other carbohydrolytic enzymes described in bifidobacteria are essential for the degradation of substrates such as α-galactosyloligosaccharides (i.e. raffinose and

74 stachyose) by α-galactosidases, oligosaccharides derived from starch and other α-
75 glucosides by α-glucosidases, and lactose and lactose-derived substrates by β-
76 galactosidases (Van der Broek, Hinz, Beldman, Vincken, & Voragen, 2008).

77 The production of organic acids, mainly acetate and lactate, as the end products of
78 carbohydrate metabolism in lactic acid bacteria (LAB) and bifidobacteria are implicated
79 in the inhibitory effect of these bacteria against Gram-negative bacteria and their
80 invasion capability of intestinal cells (Makras *et al*., 2006; Makras & De Vuyst, 2006).
81 Other mechanisms proposed for the inhibitory activity of LAB and bifidobacteria are
82 the production of hydrogen peroxide (Falagas, Betsi, & Athanasiou, 2007), bacteriocins
83 (Gillor, Etzion, & Riley, 2008), biosurfactants and other adhesion inhibitors (Velraeds,
84 van de Belt-Gritter, Busscher, Reid, & Van Der Mei, 2000), and stimulation of immune
85 response (Galdeano, de LeBlanc, Vinderola, Bonet, & Perdigón, 2007).

86 In the present work we have analyzed the fermentative metabolism of LAB and
87 bifidobacteria as a competitive advantage for growing in different carbohydrates and
88 FOS. Their glucosidase, galactosidase and fructofuranosidase activities and the
89 inhibition of enteropathogens colonization were also analyzed.

90

91 **2. Material and methods**

92 *2.1 Bacterial strains and culture conditions*

93 Lactic acid bacteria (LAB) and bifidobacteria strains used in the study were
94 *Streptococcus thermophilus* STY-31, *Lactobacillus delbrueckii* subp. *bulgaricus* LBY-
95 27, *L acidophilus* LA-5, *L. casei* LC-01 and *B. lactis* BB-12 and they were isolated from
96 a commercial symbiotic product (Tabasco et al., 2007). *S. thermophilus* was propagated
97 in ESTY broth (Pronadisa, Madrid, Spain) containing 20 g/L lactose. The different
98 strains of *Lactobacillus* and *B. lactis* were grown under anaerobic conditions (Gas-Pack,

Anaerogen; Oxoid Ltd., Hampshire, England), except for *L. casei* that was grown aerobically in MRS fermentation broth (Pronadisa), which contains neither glucose nor meat extract, supplemented with glucose (20 g/L) and L-cysteine hydrochloride (0.5 g/L). Incubations were carried out for 18-24 h at 37 ºC, excepting *L. casei* that was incubated at 30 ºC. Glucose (Pronadisa), lactose (Sharlau) and oligofructose (Raftilose P95; Orafti, Belgium) were filter-sterilized (0.45 µm) and added to the growth culture of LAB and bifidobacteria as the sole carbon source in concentration of 10 g/L. Raftilose P95 (FOS) contained 95 g/100 g fructan oligosaccharides, with degree of polymerization ranging between 2-7, and 5 g/100 g of a mixture of glucose, fructose and saccharose. The five strains were also grown on 100 g/L reconstituted skim milk supplemented with 8 g/L casein acid hydrolysate (Sharlau, Barcelona, Spain) and autoclaved at 110 °C for 10 min. FOS was added to sterilized milk at a final concentration of 20 g/L. *Escherichia coli* 0157 H^- and *Salmonella enterica* 4396 were propagated in BHI broth (Scharlau) for 24 h at 37 °C with shaking (200 rpm).

2.2 Enzyme activity assays

The enzyme activities were determined in cell-free extracts obtained from LAB and bifidobacteria strains. Bacterial cells were harvested by centrifugation (10,000 ×*g*, 15 min, 4 ºC) and washed twice with 50 mmol/L sodium phosphate buffer, pH 6.5. The harvested cells were resuspended in 500 µl 50 mmol/L sodium phosphate buffer, pH 6.5, then mixed (1:1, w/v) with sterile glass beads (Sigma–Aldrich, St. Louis, MO, USA; diameter, 150-212 µm) and beaten for mechanical disruption in a FastPrep equipment (Bio101; Savant Instruments, Holbrook, NY, USA). The insoluble fraction and glass beads were removed by centrifugation (12,000 ×*g*, 10 min, 4 ºC) and the supernatant fraction stored at –80 ºC until use. Protein content of the cell-free extracts

was determined using the Bradford method (Bradford, 1976) employing the Bio-Rad (Bio-Rad, Munich, Germany) protein assay and BSA for preparation of standard curve.

The substrates *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-α-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside (Sigma–Aldrich) were used to determine the activities α- and β-glucosidases and α- and β-galactosidases, respectively. Standard enzymatic reactions were performed by mixing 900 μL of 1 mmol/L substrate prepared in 50 mmol/L sodium phosphate buffer, pH 6.5, with 100 μL of cell-free extract (6 mg/mL) and incubating at 37 ºC. The amount of *p*-nitrophenol or *o*-nitrophenol released was monitored continuously by measuring absorbance at 410 nm in a Shimadzu UV-1601 spectrophotometer with a thermostatic CPS-240 controller (Shimadzu, Kyoto, Japan). Fructofuranosidase activity was performed as Janer et al. (2004), using 0.1 g/L FOS as substrate. Liberated fructose and glucose were determined with a commercial D-glucose/D-fructose determination kit (Roche Diagnostics, Basel, Switzerland), which allows the differentiation of both carbohydrates in a two-step enzymatic reaction. Enzymatic activities were expressed in nmol of *p*-, *o*-nitrophenol or D-fructose released from the substrate per min and mg of protein.

2.3 Organic and short-chain fatty acids (SCFA) analysis

SCFA and organic acids production by LAB and bifidobacteria were measured by following the capillary GC method described by Richardson, Calder, Srewart, and Smith (1989). For quantitative determinations, 50 μL of a solution of 2-ethylbutyric acid (100 mmol/L in methanol) was added to 0.5 mL of bacteria culture as internal standard (IS). SCFA and organic acids were extracted by the addition of 250 μL concentrated HCl and 1 mL diethyl ether followed by vortexing. After centrifugation

(3000 ×*g*, 10 min) the ether layer was removed and transferred to a GC-microvial, and 10 μL of derivatization reagent (*N*-(*tert*-butyldimethylsilyl)-*N*-*methyl*-trifluoracetamid, MTBSTFA; Fluka Chemie AG, Buch, Switzerland) was added and the mixture heated to 80 ºC for 20 min. The reaction mixture was left at room temperature for a further 24 h to ensure complete derivatization. Identification and quantification of SCFA and organic acids in the samples were based on the use of the relative response factors calculated for the target compounds in standard solutions at different concentration levels against the IS (2-ethylbutyric acid). The standard solution mixture was made from pure compounds (formate, 20 mmol/L; acetate, 20 mmol/L; propionate, 10 mmol/L; iso-butyrate, 10 mmol/L; butyrate, 10 mmol/L; valerate, 5 mmol/L; IS, 100 mmol/L; lactate, 15 mmol/L and succinate, 10 mmol/L), all purchased from Sigma–Aldrich, and derivatised in the same way than above. All measurements were made by using a minimum of triplicate assays for the same sample. SCFA and organic acids were analyzed on a Perkin-Elmer chromatograph (model 8420, Beaconsfield, UK) equipped with a FID detector and were separated using an EQUITY^{TM}-1 fused-silica capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness, Supelco, Belafonte, PA, USA). The column was held at 63 °C for 3 min after injection, temperature-programmed at 10 °C/min to 110 °C, held there for 0.5 min, and then temperature-programmed at 20 °C/min to 270 °C and held there for 15 min. The injector temperature was 300 ºC and the detector temperature was 275 ºC. Helium was the carrier gas with a column inlet pressure set at 20 psig (138 kPa) at a split ratio of 20:1 and the injection volume was 0.2 μL.

2.4 Caco-2 cell culture and inhibition of pathogen colonization assays.

Caco-2 cells were grown on Men-Alpha Medium (Invitrogen, Barcelona, Spain) supplemented with 10 mL/100 mL heat-inactivated foetal bovine serum at 37 ºC in an atmosphere containing 5% CO_2. Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest TM, Becton Dickinson, Franklin Lakes, NJ, USA) at 1×10^4 cells per well and grown during 15 days to obtain a monolayer of differentiated and polarized cells.

The inhibition of colonization of *E. coli* 0157 H^- and *S. enterica* 4396 into Caco-2 cells was evaluated by competition, exclusion and displacement mechanisms using *L. acidophilus* LA-5 in the assays. Bacteria were grown on appropriate media and cells were sedimented by centrifugation (10,000 ×*g*, for 10 min), washed twice with phosphate-buffered saline (PBS) buffer pH 7.1 (10 mmol/L Na_2HPO_4, 1 mmol/L KH_2PO_4, 140 mmol/L NaCl, 3 mmol/L KCl) and resuspended in Dulbecco´s modified Eagle medium (DMEM, Invitrogen). In the competition assay, 5×10^6 CFU/well of *L. acidophilus* LA-5 were added simultaneously with 1×10^5 CFU/well of *E. coli* or *S. enterica* and incubated for 1 h at 37 ºC in atmosphere with 5% CO_2. The number of viable *L. acidophilus*, *E. coli* and *S. enterica* used in the assays was determined by plate counting on MRS agar for *L. acidophilus* and VRBA (Scharlau) for the pathogens. In addition, Caco-2 cells were exposed to *L. acidophilus* LA-5 1 h before the addition of *E. coli* or *S. enterica* (exclusion) or 1 h after incubation of Caco-2 cells with the pathogens (displacement). Unbound bacteria to Caco-2 cells were washed off between incubations. After incubations, Caco-2 cells were washed three times in sterile PBS to remove unattached bacteria. Cells were detached from the wells by addition of 0.5 g/L trypsin-EDTA and incubation for 10 min at 37 ºC. Then, ice cold sterile PBS was added to each well followed by agitation to dissociate well contents. The bacteria bound to Caco-2 cells were quantified by serial dilutions plated on MRS agar for *L. acidophilus* and

VRBA (Scharlau) for the pathogens and incubated at 37 °C. Results were expressed as percentage of pathogen bacteria adhered to Caco-2 cells. Experimental conditions were repeated at least in triplicate.

2.5 Statistical analysis

Results are expressed as means ± standard deviation. The one-way analysis of variance (ANOVA) was performed to determine the significance of the effect of carbon sources and bacterial species on enzyme activity and SCFA production. Two-tailed Student's *t* test assuming equal variances was used to compare adhesion of pathogens tested under conditions of competition, exclusion or displacement with the adhesion of the pathogen alone. All tests were conducted using SPSS version 15.0 and a *P* value below 0.05 was considered statistically significant.

3. Results and discussion

3.1 Enzyme activities expression with different carbon sources

Differences in expression of α- and β-galactosidase, α- and β-glucosidase and fructofuranosidase activities of *S. thermophilus* STY-31, *L. delbrueckii* subsp. *bulgaricus* LBY-27, *L. casei* LC-01, *L. acidophilus* LA-5 and *B. lactis* BB-12 grown on different carbon sources have been described in this study (Table 1). *B. lactis* BB-12 showed all the enzyme activities assayed and it was the only species that exhibited β-glucosidase and α-galactosidase activities. Although *Bifidobacterium* spp. are widely recognized for the production of β-glucosidase (Van der Broek et al., 2008), it is surprising that the activity was not detected in the strains of lactobacilli tested. (Table 1). A recent study has showed that β-glucosidase activity in *Lactobacillus* strains is mostly associated to the cell-envelope (Ávila, Hidalgo, Sánchez-Moreno, Pelaez,

223 Requena, & de Pascual-Teresa, 2009). In general, the α- and β-glucosidase activities
224 increased in bacteria grown on FOS. The screening for differentially expressed genes of
225 *L. plantarum* WCFS1 has demonstrated a significant up-regulation of the α-glucosidase
226 encoding gene when the strain was grown on FOS compared to glucose (Saulnier,
227 Molenaar, De Vos, Gibson, & Kolida, 2007).

228 All the species showed β-galactosidase activity on the different carbon sources. β-
229 Galactosidase activity of all species grown on milk were higher (P <0.05) than on MRS
230 fermentation broth containing lactose as sole carbon source (Table 1). The same trend
231 was observed for the α-galactosidase activity of *B. lactis*. The increase in α- and β-
232 galactosidase activities when *B. lactis* was grown on milk was already reported in an
233 earlier study (Janer et al., 2005). The highest β-galactosidase activity among all the
234 strains studied was found in *L. acidophilus* LA-5 growing on milk. However, α-
235 glucosidase activity of *L. casei*, *L. acidophilus* and *B. lactis* and β-glucosidase activity
236 of *B. lactis* were higher (P <0.05) in cells grown on FOS than on milk.

237 Regarding the β-fructofuranosidase activity, it was only detected in *L. casei*, *L.*
238 *acidophilus* and *B. lactis* that were grown on FOS. The results obtained were 0.47, 1.43
239 and 0.50 nmol fructose released per min and mg protein, respectively. Functional
240 analyses of the FOS utilization operon in *L. acidophilus* and *L. casei* have demonstrated
241 in these species that the β-fructofuranosidase encoding gene is repressed by glucose
242 (Barrangou et al., 2003; Goh et al., 2007).

243 All the strains grew abundantly on glucose and lactose but *S. thermophilus* and *L.*
244 *delbrueckii* subsp. *bulgaricus* were not able to grow on medium with FOS. Based on
245 final counts of bacteria, a preference of lactose over glucose was detected by *B. lactis*
246 and *L. delbrueckii* subsp. *bulgaricus*. It has been described that *Bifidobacterium longum*
247 preferentially uses lactose over glucose as a carbon and energy source, since lactose led

248 to the repression of a glucose-H^+ symporter gene (Parche *et al*., 2006). Glucose-
249 nonfermenting *B. lactis* strains have been also reported (Masco et al., 2005; Van der
250 Meulen, Avonts, & De Vuyst, 2004). In our study, *B. lactis* BB-12 reached the highest
251 counts among all strains assayed when they were grown on FOS (Table 2).
252 *Bifidobacterium* spp. are generally considered the main target organisms for prebiotic
253 stimulation by inulin-type fructans (Gibson et al., 2004). Besides *B. lactis* BB-12, the
254 strains *L. acidophilus* LA-5 and *L. casei* LC-01 were the only ones that grew on FOS as
255 the only carbon source (Table 2).
256
257 *3.2 Organic acid and SCFA production*
258 The only organic acids produced by LAB and bifidobacteria grown on glucose,
259 lactose and FOS were lactate, acetate and formate. No other organic acid or SCFA were
260 produced during the growth and conditions assayed. Lactate concentration was lower on
261 FOS than on glucose and lactose in all the species excepting to *L. acidophilus,* which
262 showed the highest value ($P < 0.05$) of lactate on FOS among all the strains studied. In
263 agreement with the growth observation, *L. casei*, *L. acidophilus* and *B. lactis* produced
264 acetate using FOS as carbon source although the highest ($P < 0.05$) acetate
265 concentration was obtained by *B. lactis* on the three carbohydrates. *B. lactis* was the
266 only producer of formate using the three carbohydrates (glucose, lactose and FOS).
267 However, there was a significant ($P <0.05$) difference in the metabolite production
268 pattern in *L. casei* growing on FOS compared to glucose and lactose (Table 2). A
269 metabolic shift toward more acetate and formate production, at the expense of lactate
270 production, was observed during *L. casei* growth on FOS. The metabolic shift has been
271 also described in *Bifidobacterium* spp. grown on FOS (Falony, Lazidou, Verschaeren,
272 Weckx, Maes, & De Vuyst, 2009) and it has been associated with more ATP

273 production, resulting in a more efficient use of the available energy source (Van der
274 Meulen, Avonts, & De Vuyst, 2004).

275 Fig. 1 shows acetate and lactate production by LAB and bifidobacteria grown on
276 milk and milk supplemented with 20 g/L FOS. Significant differences ($P < 0.05$) were
277 detected between strains in the organic acid production although there were not
278 significant differences between both growth cultures. Among the strains, *B. lactis*
279 produced the highest amount of acetate ($P < 0.05$) and *L. acidophilus* the highest values
280 of lactate ($P < 0.05$). The increase of organic acids produced by LAB and bifidobacteria
281 grown on milk (Fig.1) compared with their growth on lactose as only carbon source
282 (Table 2) agreed with the higher expression of β-galactosidase activity in the strains
283 assayed (Table 1).

284

285 *3.3 Inhibition of pathogen colonization by L. acidophilus LA-5*

286 A study of inhibition to colonization was carried out among *L. acidophilus* LA-5 and
287 the adhesive enteropathogenic bacteria *S. enterica* 4396 and *E. coli* 0157H$^-$ for adhesion
288 to Caco-2 cells (Fig. 2). The strain had demonstrated the highest adhesion capacity to
289 Caco-2 cell line within the strains assayed in this study (Fernández de Palencia, López,
290 Corbí, Peláez, & Requena, 2008) and the production of an inducible bacteriocin
291 (Tabasco, García-Cayuela, Peláez, & Requena, 2009). Besides these competition
292 characteristics, *L. acidophilus* LA-5 has shown in this study the highest production of
293 lactate when grown on FOS as sole carbon source (Table 2) and on milk (Fig. 1).
294 Lactate is considered a key antimicrobial compound produced by lactobacilli (Servin,
295 2004).

296 The ability of *L. acidophilus* to displace the enteropathogens from the Caco-2 cell
297 monolayer was evaluated by calculating the percentage of the adhered enteropathogen

cells in the displacement, exclusion and competition assays compared to that obtained in assays with solely the enteropathogen. The inhibition of colonization was stronger ($P < 0.05$) in the competition assay than during exclusion and displacement assays. When the enteropathogen strains were incubated simultaneously with *L. acidophilus*, *E. coli* and *S. enterica* adhesion to the Caco-2 cells was reduced by 54% and 52%, respectively. Higher inhibition values of *S. enterica* adhesion were observed in displacement assays than in exclusion assays, that is, when Caco-2 cells were pre-incubated with the enteropathogen before addition of *L. acidophilus*. Medellin-Peña & Griffiths (2009) reported that *L. acidophilus* LA-5 produces heat-stable small molecules, possibly of proteinaceous nature, that are active against enterohemorrhagic *E. coli* O157:H7 attachment to intestinal epithelial cells. The molecule(s) reduced the transcription of enteropathogen genes involved in colonization and quorums sensing. A combined action of lactate, which acts as a permeabilizer of the Gram-negative bacterial outer membrane (Alakomi, Skytta, Saarela, Mattila-Sandholm, Latva-Kala, & Helander, 2000), and lactacin B produced by *L. acidophilus* LA-5 (Tabasco et al., 2009) could account for the inhibition of enteropathogen adhesion.

4. Conclusions

This study shows the differences in the fermentative metabolism of probiotic strains of the species *L. acidophilus*, *L. casei* and *B. lactis* when growing on different carbohydrates. The results indicate a potential benefit of adding FOS to the growth medium to increase fructofuranosidase, galactosidase and glucosidase activities and organic acid production. In addition, *L. acidophilus* LA-5 has demonstrated properties involving resistance to colonization against the intestinal pathogens *E. coli* and *S. enterica*.

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Table 1

α-, β-Glucosidase and α-, β-galactosidase activities in *S. thermophilus* STY-31, *L. delbrueckii* subsp. *bulgaricus* LBY-27, *L. casei* LC-01, *L. acidophilus* LA-5 and *B. lactis* BB-12 grown on milk and MRS fermentation broth supplemented with 10 g/L lactose or FOS. Mean values and standard deviation are indicated as nmol nitrophenol released per min and mg protein.

Species	α-Glucosidase			β-Glucosidase			α-Galactosidase			β-Galactosidase		
	Milk	Lactose	FOS	Milk	Lactose	FOS	Milk	Lactose	FOS	Milk	Lactose	FOS
S. thermophilus	–	–	ND	–	–	ND	–	–	ND	437.1(47.4)[a]	273.9(39.2)[b]	ND
L .bulgaricus	8.9(1.9)[a]	ND	ND	–	–	ND	–	–	ND	417.4(41.7)[a]	207.3(25.4)[b]	ND
L. casei	2.9(0.1)[a]	4.8(0.5)[b]	5.7(1.2)[b]	–	–	–	–	–	–	39.2(0.2)[a]	0.5(0.1)[b]	0.5(0.2)[b]
L. acidophilus	0.4(0.0)[a]	0.3(0.1)[a]	7.0(1.2)[b]	–	–	–	–	–	–	635.1(184.8)[a]	505.1(128.5)[a]	127.6(30.7)[b]
B. lactis	3.0(0.2)[a]	11.1(2.9)[b]	5.8(0.7)[c]	0.5(0.1)[a]	0.5(0.2)[a]	1.2(0.5)[b]	1.8(0.7)[a]	0.8(0.2)[b]	0.6(0.1)[b]	321.2(94.4)[a]	178.7(32.6)[b]	285.5(19.1)[a]

Values of each enzyme activity in the same row with the same superscript letter are not significantly different ($P > 0.05$); –, not detected; ND, not determined. Data are from at least three independent experiments.

Table 2

Bacterial counts (cfu/mL) and organic acids (formate, acetate and lactate) produced by *S. thermophilus* STY-31, *L. delbrueckii* subsp. *bulgaricus* LBY-27, *L. casei* LC-01, *L. acidophilus* LA-5 and *B. lactis* BB-12 after 24 h incubation in MRS fermentation broth supplemented with 10 g/L of glucose, lactose or FOS. Inoculation was of 10^7 cfu/mL for all strains. Mean values and standard deviation of organic acids are indicated as mmol/L.

Species	Glucose				Lactose				FOS			
	cfu/mL	Formate	Acetate	Lactate	cfu/mL	Formate	Acetate	Lactate	cfu/mL	Formate	Acetate	Lactate
S. thermophilus	1.2×10^9	3.5(1.0)	3.4(1.2)	59.2(10.1)	2.0×10^9	2.6(0.7)	3.1(2.3)	46.0(10.4)	2.2×10^7	–	–	7.9(1.7)*
L. bulgaricus	2.3×10^8	–	–	55.1(8.8)	8.7×10^8	–	–	58.9(7.5)	3.0×10^7	–	–	10.1(1.4)*
L. casei	2.5×10^9	–	6.6(4.0)	80.0(7.6)	2.4×10^9	–	8.2(2.9)	79.0(12.2)	5.4×10^8	6.8(2.0)	9.8(3.6)	17.2(2.7)*
L. acidophilus	2.0×10^8	–	9.5(4.8)	70.6(15.7)	2.3×10^8	–	8.3(1.1)	68.7(9.4)	1.5×10^8	–	5.6(2.5)	70.0(9.8)
B. lactis	8.4×10^8	7.7(3.5)	35.5(17.6)	16.2(3.4)	2.0×10^9	6.1(3.1)	35.3(4.2)	12.2(2.4)	9.1×10^8	5.7(0.8)	21.6(7.4)	9.7(1.5)*

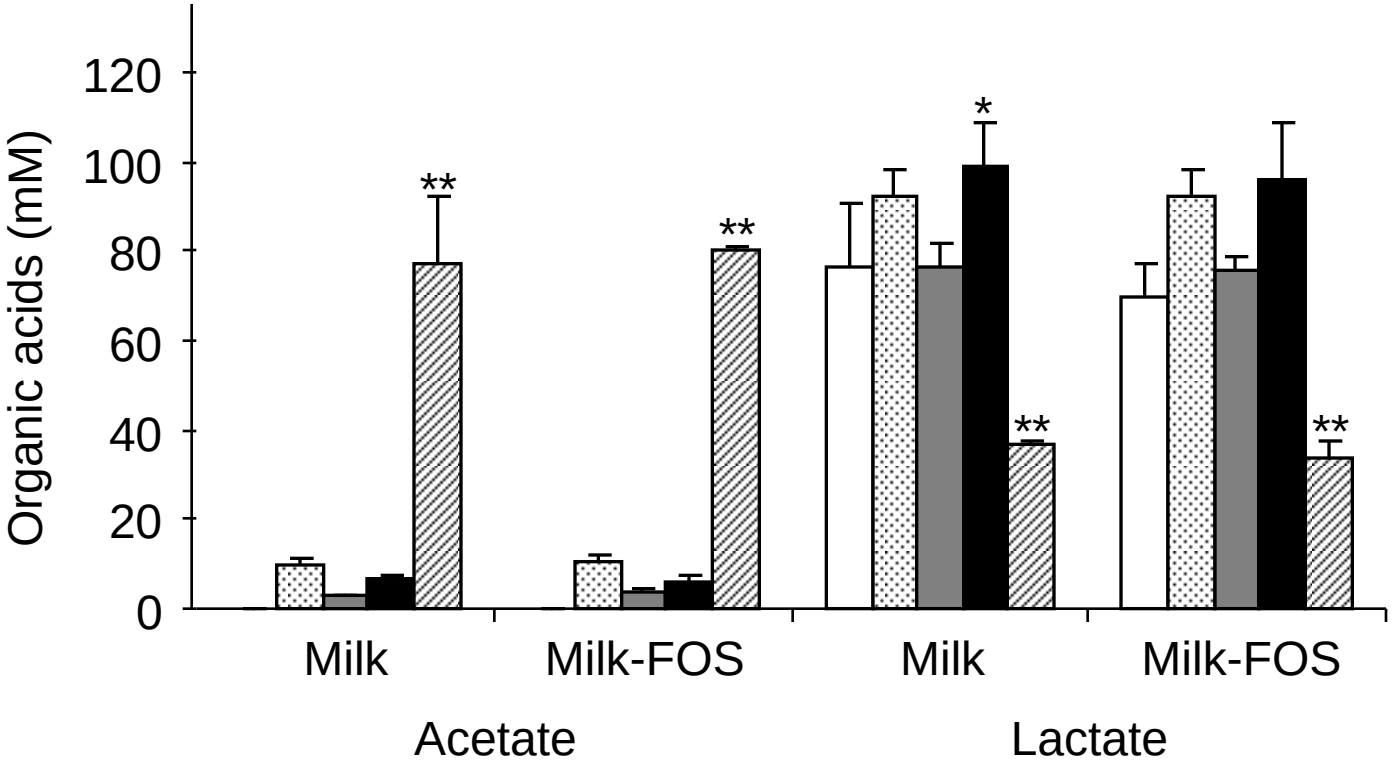
*Indicates lactate production on FOS was significantly different ($P < 0.05$) than lactate produced on glucose or lactose; –, not detected. Values are the mean of at least three independent experiments.

LEGENDS TO FIGURES

Fig. 1. Acetate and lactate concentrations (mmol/L) produced by *S. thermophilus* STY-31 (white bars), *L. casei* LC-01 (spotted bars), *L. delbrueckii* subsp. *bulgaricus* LBY-27 (grey bars), *L. acidophilus* LA-5 (black bars) and *B. lactis* BB-12 (stripped bars) on milk and milk supplemented with 20 g/L FOS after 24 h of anaerobic incubation at 37 ºC. Asterisks (* $P < 0.05$, ** $P < 0.01$) indicate the organic acid means which were significantly different between species. Values are the mean of at least three independent experiments.

Fig. 2. Adhesion (%) of *E. coli* 0157H$^-$ (black bars) and *Salmonella enterica* 4396 (white bars) to Caco-2 cells, under conditions simulating exclusion, displacement and competition with *L. acidophilus* LA-5. Statistical analysis was performed with Student´s *t* test. Asterisks (* $P < 0.05$, ** $P < 0.01$) indicate the means which were significantly different from the control, i.e. data for adhesion of *E. coli* and *S. enterica* alone. Values are the mean of at least three independent experiments.

Figure


Organic acids (mM)
120
100
80
60
40
20
0
**
**
*
**
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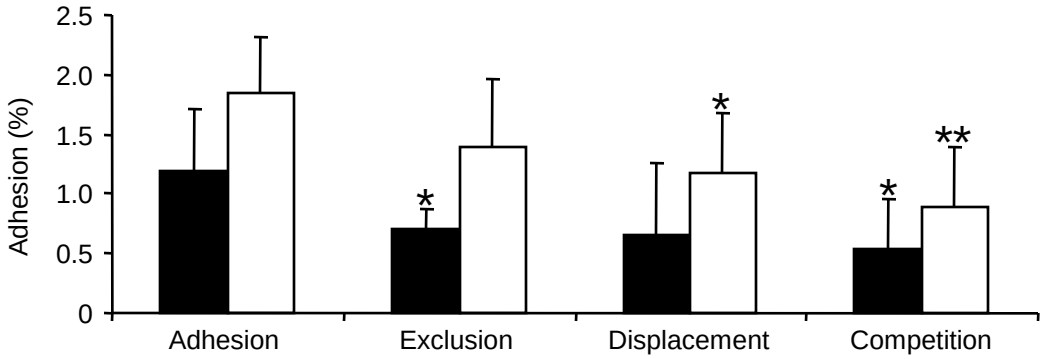
Milk
Milk-FOS
Milk
Milk-FOS
Acetate
Lactate

Fig.1

Figure


2.5
2.0
1.5
1.0
0.5
0
Adhesion (%)
*
*
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Adhesion
Exclusion
Displacement
Competition

Fig.2.